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Label-free protein profiling of formalin-fixed paraffin-embedded (FFPE) heart tissue reveals immediate mitochondrial impairment after ionising radiation

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ABSTRACT

Qualitative proteome profiling of formalin-fixed, paraffin-embedded (FFPE) tissue is advancing the field of clinical proteomics. However, quantitative proteome analysis of FFPE tissue is hampered by the lack of an efficient labelling method. The usage of conventional protein labelling on FFPE tissue has turned out to be inefficient. Classical labelling targets lysine residues that are blocked by the formalin treatment. The aim of this study was to establish a quantitative proteomics analysis of FFPE tissue by combining the label-free approach with optimised protein extraction and separation conditions.

As a model system we used FFPE heart tissue of control and exposed C57BL/6 mice after total body irradiation using a gamma ray dose of 3 gray. We identified 32 deregulated proteins ($p \leq 0.05$) in irradiated hearts 24 h after the exposure. The proteomics data were further evaluated and validated by bioinformatics and immunoblotting investigation. In good agreement with our previous results using fresh-frozen tissue, the analysis indicated radiation-induced alterations in three main biological pathways: respiratory chain, lipid metabolism and pyruvate metabolism. The label-free approach enables the quantitative measurement of radiation-induced alterations in FFPE tissue and facilitates retrospective biomarker identification using clinical archives.

Abbreviations: Gy, Gray; Mw, molecular weight; O/N, overnight; TBI, total body irradiation.

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1. Introduction

For decades, pathologists have been using formalin-fixed, paraffin-embedded (FFPE) tissue for histological analysis due to its excellent performance and suitability for long-term storage. Clinical archives including data on diagnosis and outcome may provide information on biological pathways and cellular processes leading to disease, provided that suitable molecular technologies are available [1–3].

Proteomics analysis using FFPE material as an alternative to fresh-frozen tissue has recently been investigated [4–7]. Different methods of extraction and separation of proteins from FFPE samples have been established [8–11]. However, quantitative proteomic studies on archival material have been considered as an almost impossible task, primarily due to the harsh and irreversible fixation procedures and loss of integrity during prolonged storage. The formaldehyde fixation leads to a methylol modification, tagged mainly on lysine residues [11,12]. Most chemical labels used in quantitative proteomics also target lysine residues, leading to inefficient labelling of FFPE material [13–15].

Recently, non-labelling approaches have been suggested as an alternative for quantification of FFPE proteome profiles [16,17]. The few quantitative proteomic studies published so far have been performed using tumour FFPE tissues where areas of interest are easily distinguished and dissected [18,19], thereby reducing the complexity of the resultant proteome profile.

The aim of this study was to establish a quantitative proteomics work flow using the label-free approach [20] in a tissue where no discrete target area can be observed. We have shown previously that ionising radiation has both immediate and persistent effects on the murine cardiac proteome without causing any morphological changes in the heart [21,22]. Therefore, we used the cardiac tissue of sham- and total body irradiated C57BL/6 mice as a model system. We applied the optimised protein extraction and separation conditions for FFPE tissue previously developed by us for qualitative proteomics [11]. The analysis indicated radiation-induced impairment of the cardiac mitochondrial proteome. The proteomics data were further evaluated and validated by bioinformatics and immunoblotting investigation.

2. Materials and methods

2.1. Materials

Beta-octylglucoside, SDS, and ammonium bicarbonate were obtained from Sigma (St. Louis, MO); RapiGest from Waters (USA); acetone, acetonitrile, formic acid, and trifluoroacetic acid (TFA) from Roth (Karlsuhe, Germany); dithiothreitol (DTT), iodoacetamide, tris-(hydroxymethyl) aminomethane (Tris) and sequencing grade trypsin were obtained from Promega (Madison, WI); cyano-4-hydroxycinnamic acid was obtained from Bruker Daltonik (Bremen, Germany). All solutions were prepared using HPLC grade water from Roth (Karlsuhe, Germany).

2.2. Animals

Mice (C57BL/6) were purchased from Charles River Laboratories, Germany GmbH. They were kept under standard conditions with food and water *ad libitum* (Altromin GmbH, Lage, Germany). All animal experiments were performed in compliance with the German Animal Welfare Law and were approved by the institutional committee on animal experimentation and the government of Upper Bavaria (Certificate of the Regierung von Oberbayern, No. 211-2531-54/01). Animals were irradiated using a Gamma cell 40 equipment (upper source: Cs-134 and lower source: Cs-137) (Atomic Energy of Canada Limited, Ottawa). Heart tissue obtained from male C57BL/6 mice was used for all experiments. The animals were exposed at the age of 4 months to 3 Gy total body irradiation (TBI) with a single gamma-ray dose using a dose rate of 0.6 Gy/min. Control mice were sham irradiated. The mice were sacrificed by cervical dislocation 24 h after exposure; the hearts were rapidly removed and rinsed with phosphate buffered saline to remove excess blood. A total number of 6 animals were used for this study.

2.3. Tissue preparation

Heart tissues were immediately fixed in 4% buffered formalin for 24 h, dehydrated with a graded series of ethanol before embedding in paraffin. Blocks were stored in the dark at room temperature. The blocks were cut into 20 μm sections after initial trimming to remove air exposed surfaces.

2.4. Protein extraction

Proteins were extracted as described before [11]; briefly, tissue sections (20 μm thick, 80 mm^2 wide) were placed on microscope slides and deparaffinised by incubating twice with xylene for 10 min at room temperature before rehydration in a graded series of ethanol (100%, 95% and 70%) for 10 min each. The tissue sections were scraped from the slides, washed with 0.5% beta-octylglucoside and resuspended in buffer containing 20 mM Tris–HCl, pH 8.8, 2% SDS, 1% beta-octylglucoside, 200 mM DTT, and 200 mM glycine. The extraction buffer contained protease inhibitor cocktail according to manufacturer's instructions (Roche Diagnostics).

All samples were incubated in the extraction buffer at 100 °C for 20 min, and at 80 °C for 2 h with shaking. The extracts were centrifuged for 30 min at 14,000 g at 4 °C. The protein extract was precipitated with the 2D clean up kit (GE Healthcare) following the manufacturer's instructions. The pellets were resuspended in Tris buffer in triplicate and protein quantification was done with both the Bradford method and 2D Quant Kit (GE Healthcare). All experiments were done with three biological replicates.

2.5. Protein separation and mass spectrometry

100 μg of protein extract from control and irradiated FFPE tissue was solubilised in SDS-PAGE sample buffer, and separated by 8% SDS-PAGE under reducing conditions before staining with Coomassie Brilliant Blue G-250.

The gel was cut horizontally into 5 equidistant fractions before the lanes were separated. All resultant gel slices were subjected to in-gel tryptic digestion as described previously [11] and peptides were eluted from the gel by two consecutive extractions with 50% acetonitrile (ACN)/0.5% trifluoroacetic acid (TFA) and 100% ACN/0.5% TFA and vacuum dried.